Expression of Monocyte Chemotactic Protein and Interleukin-8 by Cytokine-Activated Human Vascular Smooth Muscle Cells

Ji Ming Wang, Antonio Sica, Giuseppe Peri, Sabine Walter, Ines Martin Padura, Peter Libby, Miroslav Ceska, Ivan Lindley, Francesco Colotta, and Alberto Mantovani

The present study was designed to investigate the capacity of human vascular smooth muscle cells (SMCs) to produce a cytokine chemotactic for monocytes (monocyte chemotactic protein [MCP]) and by way of comparison, a related polypeptide activator of neutrophils (known as interleukin-8 [IL-8] or neutrophil activating protein-1 [NAP-1]). On exposure to IL-1, SMCs released high levels of chemotactic activity for monocytes, which could be removed by absorption with anti-MCP antibodies. MCP production by activated SMCs was comparable to that of IL-1–stimulated umbilical vein endothelial cells. Activated SMCs released appreciable levels of IL-8, as determined by a specific enzyme-linked immunosorbent assay, but little chemotactic activity for neutrophils. IL-1–treated SMCs expressed high levels of both MCP and IL-8 mRNA transcripts, as assessed by Northern blot analysis. Tumor necrosis factor and bacterial lipopolysaccharide but not IL-6 also induced MCP and IL-8 gene expression in SMCs. Nuclear runoff analysis revealed that IL-1 augmented transcription of the MCP and IL-8 genes. The capacity of SMCs to produce a cytokine (MCP) that recruits and activates circulating mononuclear phagocytes may be of considerable importance in the pathogenesis of vascular diseases (e.g., vasculitis and atherosclerosis) that are characterized by monocyte infiltration of the vessel wall. (Arteriosclerosis and Thrombosis 1991;11:1166–1174)
derived chemoattractant. Here, we show that on exposure to IL-1 and TNF, human vascular SMCs transcribe the MCP gene and express MCP mRNA and protein. Supernatants of activated SMCs contained appreciable levels of IL-8, as determined by enzyme-linked immunosorbent assay (ELISA), but had little chemoattract activity for neutrophils. Activated SMCs transcribed the IL-8 gene and accumulated specific mRNA. Monocyte recruitment characterizes the earliest stages of experimental atherosclerosis, and macrophages abound in regions of the advanced human atheroma. SMCs compose the bulk of blood vessels susceptible to atherosclerosis. The capacity of SMCs to express the gene for MCP may thus contribute to both the initiation and progression of atherosclerosis and other pathological processes that involve vascular or perivascular infiltration by mononuclear phagocytes.

Methods

Vascular Cells

Three separate human vascular SMC cultures were used in this study. One SMC culture (A617 from human femoral artery) was kindly donated by G. Gabbiani (University of Geneva School of Medicine, Geneva, Switzerland). The other two (Nos. 411 and 415) were SMCs isolated from outgrowths of explants of unused portions of human saphenous veins harvested for coronary artery bypass surgery as described previously. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Paisley, Scotland) that contained 5.5 mM glucose, 25 mM N'-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 10% fetal calf serum (FCS) (HyClone Laboratories, Logan, Utah). The morphology and growth pattern of the cells, as determined by phase-contrast microscopy and immunohistochemical reactivity with anti-smooth muscle actin antibody (courtesy of G. Gabbiani), were typical of cultured SMCs. Cultures were used within the 14th passage. After the cells became confluent, the SMCs were stimulated with 10 ng/ml IL-1β, washed twice with phosphate-buffered saline (PBS), and incubated again for 20 hours with DMEM containing 0.2% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Mo.), and the supernatants were collected for assessment of leukocyte chemoattract activity.

ECs (second to eighth passage) from human umbilical veins were cultured in Medium 199 (GIBCO) supplemented with 20% FCS (HyClone) and were used in the study as described in detail in previous reports.

Reagents and Cytokines

Recombinant human IL-1β (specific activity, 10⁶ units/mg) was obtained from Sclavo, Siena, Italy. Recombinant human TNF (specific activity, 8.1x10⁶ units/mg) was a kind gift from BASF/Knoll, FRG, and human recombinant IL-6 was obtained from Immunex, Seattle, Wash., through the courtesy of S. Gillis. Bacterial lipopolysaccharide (Escherichia coli 055:B5) was purchased from Difco Laboratories, Detroit, Mich.

Recombinant cytokines were endotoxin free, as measured by Limulus amoeboocyte lysate assay (M.A. Bioproducts, Walkersville, Md., sensitivity ≥1.25 endotoxin units/ml). A rabbit antiserum raised against human MCP/JE was a kind gift from B. Rollins, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Mass. JE was identified as a gene expressed in simulated mouse fibroblasts; human JE was recently shown to be identical to MCP. The antibody was used as recently described for ECs. A Sepharose–protein A–immobilized anti-MCP/JE microcolumn was prepared as described by Leonard and Skeel. The SMC supernatant was passed through the column and then assayed for chemoattractive activity for monocytes. Normal rabbit serum was used for the preparation of a control column. Actinomycin D and cycloheximide used in this study were purchased from Sigma.

Leukocyte Preparation

Human mononuclear cells were obtained from healthy volunteers by sedimentation of heparinized blood centrifuged at 400g for 20 minutes on Ficoll-Hypaque (Lymphoprep, Nyagaad, Oslo, Norway). The cells were washed, and after a differential count on a cytocentrifuge preparation, were adjusted to a concentration of 1x10⁶ monocytes/ml PBS containing 0.2% BSA. To purify the neutrophils after Ficoll-Hypaque centrifugation, the pellet containing erythrocytes and neutrophils was resuspended in PBS and mixed with a commercially available dextran solution (Eufusin, STHOLL Farmaceutici, Modena, Italy) at a concentration of 3 ml Eufusin/ml blood cells. The neutrophil-rich supernatant obtained after 30 minutes of 4°C incubation was then centrifuged, and the cell pellet was mixed with cold distilled water for 30 seconds to lyse the residual erythrocytes. Cells were then washed twice and resuspended at 1.5x10⁶/ml in Hanks’ balanced salt solution (GIBCO) with 0.2% BSA. The final preparation contained greater than 95% neutrophils.

Enzyme-Linked Immunosorbent Assay for Interleukin-8

Immunoreactive IL-8 (NAP-1) was quantified by ELISA. In brief, mouse monoclonal anti–NAP-1 antibodies were used for coating the wells of microtiter plates (for 16 hours at 4°C). After four washings (PBS [pH 7.5], containing 0.05% Tween-20), human recombinant NAP-1 (Sandoz, Basel, Switzerland) at a concentration of 0.02–10 ng/ml or unknown samples were added to precoated wells and incubated for 2 hours at 37°C. After four washings, goat anti–NAP-1 alkaline phosphatase conjugate was added, and the plates were incubated for another 2 hours at 37°C. After addition of p-nitrophenyl phosphate and further incubation, the enzymatic reac-
tion was terminated by addition of 2N NaOH. Absorbance was read at 405 nm. The detection limit of this method is about 3.0 pg/ml.

Chemotaxis Assay

Leukocyte chemotaxis was assessed by a microchamber technique. Thirty-five-microliter supernatants diluted in PBS (for monocytes) or in Hanks' balanced salt solution (for neutrophils) were placed in the lower compartment of the microchamber, and 50 μl cell suspension was seeded in the upper compartment. The two compartments were separated by a polycarbonate filter (5-μm pore size, Neuroprobe, Cabin John, Md.; polyvinylpyrrolidone-free for neutrophil migration). The chambers were incubated at 37°C for 90 minutes (for monocytes) or 120 minutes (for neutrophils); at the end of incubation, filters were removed, fixed, and stained with Diff-Quik (Harleco, Gibbstown, N.J.), and five oil-immersion fields were counted after the samples were coded. Each experiment used N-formyl-methyl-leucyl-phenylalanine (Sigma) as a reference chemoattractant at the optimal concentration of 10 nM. The statistical significance of migration toward stimulus versus that toward medium control was assessed by Dunnet's test. One unit per milliliter activity was defined as the reciprocal of the dilution at which 50% of maximal chemotactic response was obtained compared with that obtained toward N-formyl-methyl-leucyl-phenylalanine.

Northern Blot Analysis

Northern blot analysis was performed according to standard procedures. Total RNA was isolated by the guanidine isothiocyanate method. Fifteen micrograms total RNA was analyzed by electrophoresis through 1% agarose formaldehyde gels, followed by Northern blot transfer to Gene Screen Plus membranes (New England Nuclear, Boston, Mass.). The plasmids containing the full-length cDNA human MCP probe (0.672-kb Pst I-Pst I fragment42) and the human IL-8 cDNA clone (0.3-kb Pst I-EcoR1 fragment) were nick translated with α-phosphorus-32-labeled deoxycytidine triphosphate (5,000 Ci/mmol; Amersham, Buckinghamshire, UK). Membranes were pretreated and hybridized in 50% formamide.
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Bar graphs showing production of monocyte and neutrophil chemotactic activity in vascular cells induced by interleukin-1 (IL-1). Vascular cells were incubated with IL-1β for 4 hours and then washed, and supernatants were harvested 20 hours later. Chemotactic activity is expressed as units per milliliter (±SEM of five separate experiments). One unit per milliliter was defined as the reciprocal of the dilution at which 50% of the maximum chemotactic response was obtained compared with that produced by N-formyl-methyl-leucyl-phenylalanine. Panel A: Monocyte chemotactic activity (MCP). Panel B: Neutrophil chemotactic activity (IL8). (−), Without IL-1.

(Merck, Rahway, N.J.) with 10% dextran sulfate (Sigma), washed twice with 2× standard saline citrate (SSC) (1×SSC is 0.15 M NaCl, and 0.015 M sodium citrate), twice with 2×SSC plus 1% sodium dodecyl sulfate (SDS, Merck) at 60°C for 30 minutes, and finally twice with 0.1×SSC at room temperature for 30 minutes. The membranes were exposed for 12–24 hours at −80°C with intensifying screens. RNA loading and transfer to membranes was checked by examination under ultraviolet light and hybridization of the blot with an α-actin probe.

Bar graph showing absorption of monocyte chemotactic activity induced in smooth muscle cells (SMCs) by anti-JE antisem (●). A Sepharose-protein A–immobilized anti-MCP/JE microcolumn was prepared according to Leonard and Skeel.11,38 Interleukin-1-stimulated SMC supernatant was passed through the column and tested for monocyte chemotactic activity at various dilutions. Normal rabbit serum was used for the control column. Chemotactic activity was expressed as number of migrated monocytes at a 1:15 dilution of the SMC supernatant (●). MCP, monocyte chemotactic protein.

Line plot showing production of interleukin-8 (IL-8, ng/ml) measured by an enzyme-linked immunosorbent assay by IL-stimulated smooth muscle cells (SMCs). SMC data (●) comprise six separate experiments, whereas endothelial cell (EC) results (○) are from one experiment.
FIGURE 5. Northern blots showing cytokine induction of monocyte chemotactic protein (MCP) (upper panel) and interleukin-8 (IL-8) mRNA (lower panel) in smooth muscle cells. Total RNA was extracted from unstimulated smooth muscle cells (lane 1) or smooth muscle cells incubated with 10 ng/ml IL-1 for 30 minutes (lane 2), 2 hours (lane 3), or overnight (lane 4); lipopolysaccharide (10 μg/ml, 4 hours; lane 5); tumor necrosis factor (400 units/ml, 4 hours; lane 6); or IL-6 (200 units/ml, 4 hours; lane 7). IL-1-treated (10 ng/ml, 4 hours) endothelial cells (lane 8) were used as a positive control. 28S and 18S denote 28S and 18S subunits of rRNA. α-Actin, blot of α-actin probe.

and deoxyadenosine triphosphate) and 100 μCi α-phosphorus-32-labeled uridine triphosphate (Amersham, 6,000 Ci/mmol) were added to 230 μl nuclei suspension and incubated at 30°C for 30 minutes. Elongated transcripts were then isolated by use of the guanidine/CsCl procedure as described above, with 50 μg yeast tRNA added as a carrier. The RNA pellet was resuspended in 180 μl ice-cold buffer (0.5 M Tris HCl [pH 8] and 1.5 M NaCl) and denatured by addition of 20 μl 2N NaOH on ice for 10 minutes. The solution was neutralized by addition of HEPES (pH 7.2, final concentration, 0.48 M). RNA was then precipitated after addition of 880 μl ethanol; the pellet was resuspended in 100 μl hybridization solution (10 mM N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 0.2% SDS, 10 mM EDTA, and 3 M NaCl), and the radioactivity was checked with a beta counter. The RNA solution was hybridized at 65°C for 48 hours to DNA immobilized on nitrocellulose filters. In a given experiment each filter was hybridized with RNA solutions of equal radioactivity. The filters were then washed with several changes of 0.2×SSC at 65°C for 30 minutes and incubated at 37°C in 0.2×SSC with 10 μg/ml RNase A for 30 minutes. Filters were then exposed for autoradiography as described above. For immobilization of DNA to the filters, 5 μg plasmids containing MCP or IL-8 cDNA were denatured with 0.3 M NaOH at 60°C for 30 minutes, neutralized with ammonium acetate (final concentration, 4 M), and spotted onto a nitrocellulose filter (Schleicher and Schuell, Dassel, FRG) by use of a slot-blot apparatus (Schleicher and Schuell). pBR 322 DNA was used as a negative hybridization control.
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**Figure 6.** Northern blots showing effect of simultaneous administration of interleukin-1 (IL-1) and actinomycin D on monocyte chemotactic protein mRNA expression in smooth muscle cells (SMCs). RNAs were isolated from untreated SMCs (lane 1) or SMCs treated with IL-1 (10 ng/ml, 4 hours; lane 2), cycloheximide (10 μg/ml, 4 hours; lane 3), actinomycin (5 μg/ml, 4 hours; lane 4), actinomycin D and IL-1 (4 hours; lane 5), and cycloheximide and IL-1 (4 hours; lane 6). α-Actin blot of α-actin probe.

Quantification of Autoradiography

The optical density of the autoradiographic bands integrated for the band area was evaluated by a digitized image analyzer (RAS 3000, Loats System, Amersham). The "relative induction" was determined by dividing the densitometric quantification of the induced bands by the densitometric quantification of the unstimulated band.

**Results**

On exposure to IL-1β, vascular SMCs released considerable amounts of monocyte chemotactic activity, as assessed by induction of monocyte migration across polycarbonate filters. Figure 1 shows data from one experiment (representative of five performed), in which SMCs were exposed to 10 ng/ml IL-1β; the supernatant was obtained after 20 hours. Figure 2 presents data expressed as units per milliliter of chemotactic activity. In this series of five experiments, IL-1-stimulated SMCs released 53±14 units/ml (mean±SEM; range, 27–100 units/ml) monocyte chemotactic activity compared with 3±1 units/ml (range, 0.5–7 units/ml) from unstimulated SMCs (p<0.01). Amounts of MCP released by activated SMCs were comparable to those produced by human umbilical vein ECs stimulated under the same conditions (References 10 and 12 and Figures 1 and 2). Several lines of evidence indicate that the IL-1 molecule per se was not responsible for monocyte chemotaxis. When SMCs were exposed to 10 ng/ml IL-1β for 4 hours, washed, and then incubated with serum-free medium for 20 hours, they released high levels of MCP. Moreover, in agreement with previous reports,9,12,19 IL-1 tested over a wide range of concentrations and experimental conditions affected neither leukocyte migration nor the chemotactic activity of reference chemotactants (data not shown).

Another series of experiments examined the kinetics of MCP activity and the effect of different IL-1 doses. The maximum induction of MCP activity in SMCs by IL-1 occurred after 4 hours' incubation, and the optimal doses for the stimulation ranged between 1 and 10 ng/ml in different experiments (data not shown).

To identify MCP as the mediator responsible for SMC-produced chemotactic activity, we used a specific antiserum raised against MCP/JE.11 Absorption with immobilized anti-MCP/JE antibodies reduced SMC chemotactic activity for monocytes by 70% (Figure 3). Nonimmune serum had no effect, and the antiserum alone did not affect the chemotactic activity of N-formyl-methyl-leucyl-phenylalanine, excluding nonspecific effects.

By way of comparison, we examined IL-8 production by cytokine-stimulated SMCs by use of an ELISA and chemotaxis for neutrophils. As shown in Figure 4, IL-1-stimulated SMCs released appreciable levels of IL-8 as determined by ELISA. In apparent contrast with these data, supernatants of IL-1-stimulated SMCs had little chemotactic activity for neutrophils (Figures 1 and 2). The results shown in Figure 1 are representative of three separate vascular SMC lines tested over a period of 6 months. By way of comparison, Figure 1 also shows the release of chemotactants by IL-1-stimulated umbilical vein ECs. As reported previously,9,11 cytokine-activated ECs released both IL-8 and MCP. The MCP activity released by SMCs and ECs was comparable.

In an effort to define the molecular basis of MCP activity, we examined expression of cytokine mRNA transcripts in SMCs by Northern blot analysis. Unstimulated SMCs did not express appreciable levels of steady-state MCP and IL-8 transcripts (Figure 5),

![Figure 7.](http://atvb.ahajournals.org/) Nuclear runoff analysis of monocyte chemotactic protein (MCP) and interleukin-8 (IL-8) gene expression in smooth muscle cells (SMCs) induced by IL-1. DNA samples applied to filters were as follows (at left, from top to bottom): human MCP cDNA, human IL-8 cDNA, IL-6 cDNA, pBR322, and actin. Radioactive transcripts were obtained from untreated SMCs (SMC [-]) and IL-1β-treated (10 ng/ml, 3.5 hours) SMCs (SMC+IL-1 3.5h). Densitometric analysis revealed a fourfold increase of MCP transcription.
although some gene transcription was detectable by
runoff analysis (see below). On exposure to IL-1,
high levels of MCP and IL-8 mRNA were detected as
early as 120 minutes, with maximum expression at
4–20 hours. The levels of expression in activated
SMCs were comparable to those observed in IL-1-
stimulated ECs used as reference controls in these
experiments (Figure 5). TNF and lipopolysaccharide
but not IL-6 also induced cytokine gene expression in
SMCs (Figure 5). Induction of MCP mRNA expres-
sion was not affected by exposure to IL-1 in the
presence of the protein synthesis inhibitor cyclohex-
emide, whereas the inhibitor of transcription actino-
mycin D completely blocked this process. Actually,
cycloheximide alone induced detectable levels of
MCP mRNA and augmented induction by IL-1 (Fig-
ure 6). Superinduction by inhibition of protein syn-
thesis may imply the existence of labile inhibitors
of transcription of the MCP gene. To evaluate more
directly the role of gene transcription in accumula-
tion of steady-state MCP and IL-8 transcripts, we
performed nuclear runoff analyses. IL-1 induced a
fourfold augmentation of MCP gene transcription in
vascular SMCs (Figure 7). A similar increase of gene
transcription was detected with IL-8. The half-life of
MCP transcripts estimated by exposing cells to acti-
nomycin D after IL-1 induction exceeded 2 hours
(Figures 6 and 8). We could not precisely define the
half-life of MCP mRNA because more prolonged
exposure of IL-1–treated cells to actinomycin D led
to gross loss of cell viability.

Discussion

These results demonstrate that SMCs, on stimula-
tion with IL-1, produce high levels of MCP. MCP
release induced by IL-1 in SMCs was associated with
expression of specific mRNA transcripts. Nuclear run-
off experiments revealed that IL-1 transcriptionally
activates the MCP gene. Previous studies had already
shown that baboon aortic SMCs in the absence of
deliberate stimulation produce an 8–12-kd monocyte
chemoattractant,33,34 and more recently this has been
shown to be antigenically cross-reactive with a
chemoattractant produced by tumor cell lines.35 The
results reported here which identify MCP as a product
released by SMC on IL-1 activation, provide a molecu-
lar basis for these observations.

It has been previously reported9–12 and confirmed
here that ECs express MCP (and IL-8) both at the
mRNA and at the protein levels. With the caveat that
we studied vascular SMCs and ECs from different
blood vessels and that a quantitative comparison was
beyond the scope of the present study, the results
obtained indicate comparable levels of production of
MCP for ECs and SMCs.

In an effort to comprehensively characterize the
production of chemotactic cytokines by SMCs, we
studied the expression and release of IL-8. Cytokine-
stimulated SMCs transcribed the IL-8 gene and
expressed high levels of steady-state IL-8 mRNA
transcripts. As expected on the basis of these results,
IL-8 was detected in the supernatants of activated
SMCs by ELISA, but levels of neutrophil chemotac-
tic activity were negligible. The coexistence of an
inhibitor or the production of a version of IL-8 with
lower specific activity could account for this apparent
discrepancy. In preliminary experiments we failed to
identify inhibitory activity for neutrophil chemotaxis
in SMC supernatants. The occurrence of IL-8 mole-
cules with different N-termini has been documented
(e.g., for review, see Reference 44), and a 77 amino
acid version of IL-8 has been reported to be 10 times
less active than the "conventional" 73 amino acid
form.45 Elucidation of this point, which was beyond
the main objective of the present study, will require
identification of the IL-8 form produced by SMCs.

Renal mesangial cells share several properties with
SMCs.46 In a recent analysis of cytokine production
by human renal mesangial cells, we found a pattern
of chemotactic cytokine release similar to that re-
ported here for vascular SMCs, that is, release of
MCP in the absence of appreciable levels of neutro-
phil chemotactic activity (C. Zoja et al, unpublished
observations); gene transcription and steady-state
mRNA transcripts of both MCP and IL-8 were
detected in stimulated human mesangial cells. MCP
production by SMC-related mesangial cells may play
an important role in kidney pathology.
SMCs are a major constituent of blood vessels. Cells of the monocyte-macrophage lineage have been identified in and around vessel walls affected by vasculitis. Monocyte extravasation represents an early event in atherosclerosis. Minimally modified low density lipoproteins, which may play an important role in atherosclerosis, induce expression of colony-stimulating factors in vascular endothelium. These modified lipoproteins also induce release of monocyte chemotactic activity by rabbit endothelium. While this report was being submitted, MCP expression was shown to underlie the latter observation. In this context, it is of interest that Taubman et al recently reported that platelet-derived growth factor and angiotensin II have opposing influences on JE mRNA levels in SMCs, causing increased and decreased expression, respectively. The capacity of SMCs to produce a cytokine (MCP) that recruits and activates circulating mononuclear phagocytes may be of considerable importance in the pathogenesis of vascular diseases characterized by monocyte infiltration of the vessel wall.

References

32. Bottazzi B, Colotta F, Sica A, Nobili N, Mantovani A: A chemotactant expressed in human sarcoma cells (tumor-
derived chemotactic factor, TDCF) is identical to monocyte chemoattractant protein-1/monocyte chemotactic and activating factor (MCP-1/MCAF).


46. Striker GE, Striker L: Glomerular cell culture. Lab Invest 1985;53:122–131


**KEY WORDS** • smooth muscle cells • monocyte chemotactic factor • interleukin-1 • tumor necrosis factor • atherosclerosis • vasculitis

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*Arterioscler Thromb Vasc Biol.* 1991;11:1166-1174
doi: 10.1161/01.ATV.11.5.1166

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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